

## MEMBER OF THE TNF LIGAND FAMILY

The present invention relates to a novel protein of the TNF ligand superfamily, nucleotides coding for it, vectors and host cells containing the same and methods of screening for modulators of the interaction between said protein and its receptor, said modulators for use in therapy for various disorders including, but not restricted to, cancer, inflammation, infection and autoimmune disease. Also, direct use of said ligand in therapy, for example against viral diseases or as a potential vaccine adjuvant.

Fifteen other members of the TNF ligand family have currently been cloned and published and most have been shown to bind to cell-surface receptors of the TNF receptor family. The interaction between a TNF ligand and its receptor is the key signal to start a chain of events leading to a range of responses as diverse as T-cell proliferation, apoptosis and induction of cytokine production. Some activities such as induction of T-cell proliferation are common to many members of the family, whilst some are shared by only a few, and others are unique. The interaction between these ligands and their receptors provides an attractive target for the development of novel therapies.

The present invention provides an isolated protein comprising i) a polypeptide having the amino acid sequence of figure 1 or ii) a variant of the polypeptide of i). The invention also provides an isolated protein comprising a polypeptide having the amino acid sequence as provided in figure 2 or variants thereof. The protein having the amino acid sequence provided in Figure 2 is obtainable from humans and is a type II membrane protein with a single transmembrane domain near the N-terminus, which contains two potential N-linked glycosylation sites, and a protease cleavage site between amino acids arginine 133 and alanine 134. The polypeptide having the amino acid sequence provided in figure 1 is soluble, and forms part of the extracellular region of the polypeptide having the amino acid sequence provided in figure 2. Preferably the protein of the invention comprises a polypeptide which is 65%, preferably 75%, more preferably 80% and even more preferably 90% homologous to the amino acid sequence of Fig 1. The protein of the invention most preferably comprises a polypeptide which is, at least 95%, for example 97%, 98% or 99% homologous to the amino acid

sequence of Fig 1. Preferably the protein of the invention is obtainable from mammals, more preferably from mice or humans, and most preferably from humans.

5 The present invention further provides a protein comprising a polypeptide which has the sequence as provided in figure 2 from amino acid 134 onwards, or the sequence as provided in figure 6 from amino acid 127 onwards.

10 The present invention further provides a protein comprising a polypeptide which has the sequence as provided in figure 2 from amino acid 122 onwards.

The present invention further provides a protein comprising a polypeptide which has the sequence as provided in figure 6 from amino acid 115 onwards.

15 The present invention further provides an isolated protein comprising a polypeptide having the amino acid sequence as provided in figure 5, or variants thereof. Moreover, the invention provides an isolated protein comprising a polypeptide having the amino acid sequence as provided in figure 6 or variants thereof. The protein having the amino acid sequence as provided in figure 6 is  
20 isolatable from mice and is a type II membrane protein with a single transmembrane domain near the N-terminus, which protein contains one potential N-linked glycosylation site, and a protease cleavage site between amino acids arginine126 and alanine127. The polypeptide having the amino acid sequence provided in figure 5 is soluble, and forms part of the extracellular  
25 region of the polypeptide having the amino acid sequence provided in figure 6.

Proteins of the invention isolatable from humans, and proteins of the invention isolatable from mice are highly homologous, displaying 67% amino acid identity over their entire sequence. In the C-terminal region involved in receptor binding,  
30 amino acid identity is much higher (87%). A significant difference between proteins of the invention isolatable from humans or mice is the presence of an additional exon in the mouse sequence encoding an extra 31 amino acids which reduces the overall homology between the two proteins.

The term variant refers to proteins which have substantially the same biological functionality as the protein for which sequence information has been provided. The term variant encompasses fragments, derivatives and analogues of the protein of the invention.

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Fragments include portions of the protein which retain sufficient identity to the original protein to be effective for example in a screen.

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Derivatives include alternate forms of the protein sequence which may have deletions, additions or substitutions of one or more amino acids. It will be understood by a person skilled in the art that certain substitutions, deletions or additions of amino acids can be made, or indeed can occur naturally, without substantially altering the function of the protein.

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Analogues include but are not limited to precursor proteins which can be activated by cleavage of the precursor protein to produce an active mature protein, or a fusion with a leader or secretory sequence to aid purification.

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The protein of the present invention may be a recombinant protein, a natural protein or a synthetic protein.

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The proteins of the invention may be present in all embodiments in trimeric form and such trimers form an embodiment of the invention. Typically the proteins of the invention will bind to their receptor as a trimer, thus allowing two or more receptor molecules to be brought into proximity. A trimer may be a heterotrimer wherein more than one type of subunit is present, or a homotrimer wherein all subunits are the same.

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The present invention also provides antibodies specific for the protein of the invention. The term antibody as used herein includes all immunoglobulins and fragments thereof which contain recognition sites for antigenic determinants of proteins of the present invention. The antibodies of the present invention may be polyclonal or monoclonal, may be intact antibody molecules or fragments containing the active binding region of the antibody, e.g. Fab or F(ab)<sub>2</sub>. The present invention also includes chimeric, single chain and humanised antibodies

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and fusions with non-immunoglobulin molecules. Various procedures known in the art may be used for the production of such antibodies and fragments.

5 The proteins of the invention, their variants or cells expressing them can be used as an immunogen to produce antibodies thereto. Antibodies generated against the proteins of the invention can be obtained by direct injection of the polypeptide into an animal, preferably a non-human. The antibody so obtained will then bind the protein itself. In this manner, even a fragment of the protein of the invention can be used to generate antibodies binding the whole native  
10 protein.

The antibodies of the present invention may be used to locate the protein of the invention in tissue expressing that protein. They are also, for example, useful for purification of a protein of the invention, and accordingly there is provided a  
15 method of purifying a protein of the invention which method comprises the use of an antibody of the present invention. The antibodies of the present invention may also be used as therapeutic agents in their own right.

A further aspect of the invention provides an isolated polynucleotide which  
20 encodes a protein of the invention. Also included within the invention are anti-sense nucleotides or complementary strands. Preferably the nucleotide encodes a protein of the invention isolatable from a mouse or a human. More preferably the isolated polynucleotide comprises the polynucleotide portion having the nucleotide sequence shown in figure 3, which codes for the polypeptide shown  
25 in Figure 1, a variant of said portion, or a complementary strand. The present invention further provides an isolated polynucleotide comprising the nucleotide sequence shown in figure 4, which codes for the polypeptide of Figure 2.

The nucleotide sequence may be isolated from a cell (preferably a human cell),  
30 by screening with a probe derived from the protein of the invention, or by other methodologies known in the art such as polymerase chain reaction (PCR) for example on genomic DNA with appropriate oligonucleotide primers derived from or designed based on the protein of the invention. A bacterial artificial chromosome library can be generated using mouse or human DNA for the  
35 purposes of screening.

The nucleotide sequences of the present invention may be in the form of RNA or in the form of DNA, for example cDNA, genomic DNA, and synthetic DNA. Preferably the nucleotide sequence of the invention is cDNA. The DNA may be  
5 double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the protein of the invention may be identical to one of the coding sequences set forth in the Figures, or may be a different coding sequence which as a result of the redundancy or degeneracy of the genetic code, encodes the same protein as  
10 the sequences set forth therein.

A nucleotide sequence which encodes a protein of the present invention may include: a coding sequence for the protein or any variant thereof; a coding sequence for the protein or any variant thereof and additional coding sequence  
15 such as a leader or secretory sequence or a proprotein sequence; a coding sequence for the protein or any variant thereof (and optionally additional coding sequence) and non-coding sequences, such as introns or non-coding sequences 5' and/or 3' of the coding sequence for the full length protein.

The invention also provides nucleotide variants, analogues, derivatives and fragments which encode a protein of the invention. Nucleotides are included which preferably have at least 65% identity over their entire length to the nucleotide having the sequence of Figure 3. More preferred are those sequences which have at least 75% identity over their entire length to the  
25 nucleotide having the sequence of Figure 3. Even more preferred are polynucleotides which demonstrate at least 90%, for example 95%, 97%, 98% or 99% identity over their entire length to the nucleotide having the sequence of Figure 3.

The nucleotide sequences of the invention may also have the coding sequence fused in frame to one or more marker sequences which allow for purification of the protein of the present invention such as a FLAG epitope, a myc sequence, or a secretory signal.

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The nucleotide sequences of the present invention may be employed for producing a protein of the invention by recombinant techniques. Thus, for example the nucleotide sequence may be included in any one of a variety of expression vehicles or cloning vehicles, in particular vectors or plasmids for  
5 expressing a protein. Such vectors include chromosomal, non-chromosomal and synthetic DNA sequences. Examples of suitable vectors include derivatives of bacterial plasmids; phage DNA; yeast plasmids; vectors derived from combinations of plasmids and phage DNA and viral DNA. However, any other plasmid or vector may be used as long as it is replicable and viable in the host.

10 More particularly, the present invention also provides a vector comprising one or more of the nucleotide sequences as described above. The vectors are, for example, an expression vector, such as a plasmid or viral vector into which an isolated polynucleotide of the invention has been inserted, in a forward or  
15 reverse orientation. In a preferred aspect of this embodiment, the vector further comprises one or more regulatory sequences to direct mRNA synthesis, including, for example, a promoter, operably linked to the sequence. Suitable promoters include: CMV, LTR or SV40 promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses.  
20 The vector may contain an enhancer and a ribosome binding site for translation initiation and a transcription terminator.

Large numbers of suitable vectors and promoters/enhancers, will be known to those of skill in the art, but any plasmid or vector, promoter/enhancer may be  
25 used as long as it is replicable and functional in the host.

Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts include mammalian expression vectors, insect expression vectors, yeast expression vectors, bacterial expression vectors and viral  
30 expression vectors and are described in Sambrook et al., Molecular Cloning: A laboratory Manual, Second Edition, Cold Spring Harbor, NY., (1989) A preferred vector is pFLAG-CMV-1 or pcDNA3.

The vector may also include appropriate sequences for selection and/or  
35 amplification of expression. For this the vector will comprise one or more

phenotypic selectable/amplifiable markers. Such markers are also well known to those skilled in the art.

5 In a further embodiment, the present invention provides host cells comprising a vector of the invention, and capable of expressing a nucleotide sequence of the invention. The host cells can be, for example, a higher eukaryotic cell, such as a mammalian cell or a lower eukaryotic cell, such as a yeast cell or a prokaryotic cell such as a bacterial cell. Suitable prokaryotic hosts for transformation include E-coli. Suitable eukaryotic hosts include HEK293T cells and HeLa cells.

10 Cell free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

15 Routine methods can be employed to purify the protein of the invention from recombinant cell cultures. Such methods are well understood by persons skilled in the art.

20 The proteins and nucleotide sequences of the present invention are provided in an isolated form. The term "isolated" is intended to convey that the material is not in its native state. Thus, the naturally-occurring nucleotide sequence or protein present in a living animal is in its native state and is not isolated, but the same nucleotide sequence or protein, separated from some or all of the materials it co-exists with in the natural system, is isolated. Similarly, a protein which has been produced by synthetic means, for example, by recombinant methods is "isolated." Such nucleotide sequence could be part of a vector. Such nucleotide sequence or protein could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment. The proteins and nucleotide sequences of the present invention are also preferably provided in purified form, and preferably are purified to at least 50% purity, more preferably about 75% purity, most preferably 90% purity or greater, such as 95%, 98% pure.

30 A further aspect of the present invention is the use of the proteins according to the invention in screening methods. Such methods identify compounds which act as modulators of the interaction between proteins of the invention and their

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receptor. In general terms, such screens will comprise contacting a protein of the invention, preferably in trimeric form, and its receptor in the presence or absence of the test compound, and measuring the increase or decrease in the level of binding, or increase or decrease in a response, for example NF- $\kappa$ B activation or CD40 activation, when the test compound is present. The proteins of the invention may be used in high throughput screens, thus enabling large numbers of compounds to be studied. The screening methods of the invention are generally well known to persons skilled in the art. The present invention also includes within its scope those compounds which are identified by the screening methods of the invention as possessing useful activity.

The present invention further provides compounds which are modulators of the interaction between a protein of the invention and its receptor for use in therapy, for example immunotherapy. The compounds are provided for use in the treatment of, for example, autoimmune disease, inflammation and other diseases associated with the activation of the transcription factor NF- $\kappa$ B, for example, rheumatoid arthritis, neuronal inflammation, asthma, in the treatment of cancers, in the treatment of infections, such as septic shock and in the treatment of atherosclerosis. The compounds may be agonists or antagonists of the receptor to which the proteins of the invention bind, but preferably are antagonists. The compounds include, for example, aptamers, polypeptides and small molecules.

The invention further provides the use of compounds which have been identified by the screening techniques of the invention, for the manufacture of a medicament for use in treatment or prophylaxis of disorders that are responsive to modulation of the interaction between the protein of the invention and its receptor.

The present invention additionally provides a method of treatment of a disorder which is responsive to modulation of the interaction between the protein of the invention and its receptor which comprises administering to a patient an effective amount of a compound identifiable by the screening techniques of the invention, or an effective amount of the protein of the invention.



The present invention further provides the protein of the invention for use in therapy, for example, for use in immunotherapy, particularly during viral infections, as a vaccine, or as a vaccine adjuvant.

5 The present invention also provides the use of the protein of the invention in the manufacture of a medicament for use in immunotherapy, for example, during viral infections, or as a vaccine adjuvant.

10 The present invention additionally provides a method of treatment of a disorder which is responsive to an increased amount of the protein of the invention which comprises administering to a patient an effective amount of the protein of the invention.

15 The invention also provides a nucleotide sequence as defined herein, for use in gene therapy or as a vaccine, for example, to increase the production of the protein of the invention in disorders which respond to an increased level of the protein of the sequence of Fig 1 or Fig 2. A patient may be provided with said nucleotide as a naked polynucleotide in the form of an expression vector such as a plasmid, or with a viral vector comprising said nucleotide, or a cell comprising  
20 said nucleotide or vector.

Complementary or anti-sense strands of the nucleotide sequences of the invention can also be used in gene therapy. For example, a cDNA sequence or fragments thereof could be used in gene therapy strategies to down-regulate  
25 expression of the protein of the invention. Antisense technology can be used to control gene expression through triple-helix formation of antisense DNA or RNA, both of which methods are based on binding of a nucleotide sequence to DNA or RNA.

30 Suitable techniques for introducing the naked polynucleotide or vector into a patient include topical application with an appropriate vehicle. The naked polynucleotide or vector may be present together with a pharmaceutically acceptable excipient, such as phosphate buffered saline (PBS). One technique involves particle bombardment (which is also known as 'gene gun' technology

and is described in US Patent No. 5371015). Here inert particles (such as gold beads) are coated with a nucleic acid, and are accelerated at speeds sufficient to enable them to penetrate a surface of a recipient (e.g. skin), for example by means of discharge under high pressure from a projecting device. (Particles  
5 coated with a nucleic acid molecule of the present invention are within the scope of the present invention, as are devices loaded with such particles.) Other methods of administering the nucleic acid directly to a recipient include ultrasound, electrical stimulation, electroporation and microseeding which is described in US-5,697,901.

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Nucleic acid molecules of the present invention may also be administered by means of specialised delivery vectors useful in gene therapy. Gene therapy approaches are discussed for example by Verme *et al*, Nature 1997, 389:239-242. Both viral and non-viral systems can be used. Viral based systems include  
15 retroviral, lentiviral, adenoviral, adeno-associated viral, herpes viral and vaccinia-viral based systems. Non-viral based systems include direct administration of nucleic acids and liposome-based systems.

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A nucleic acid sequence of the present invention may be administered by means  
20 of transformed cells. Such cells include cells harvested from a subject. The naked polynucleotide or vector of the present invention can be introduced into such cells *in vitro* and the transformed cells can later be returned to the subject. The polynucleotide of the invention may integrate into nucleic acid already present in a cell by homologous recombination events. A transformed cell may,  
25 if desired, be grown up *in vitro* and one or more of the resultant cells may be used in the present invention. Cells can be provided at an appropriate site in a patient by known surgical or microsurgical techniques (e.g. grafting, micro-injection, etc.)

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The invention also relates to compositions comprising the polypeptide, polynucleotide, vector or transfected cell of the invention in addition to those which

may be administered by gene gun. Thus, the polypeptides of the present invention may be employed in combination with a non-sterile or sterile carrier or carriers for use with cells, tissues or organisms, such as a pharmaceutical carrier suitable for administration to a subject. Such compositions comprise, for instance, a media  
5 additive or a therapeutically effective amount of a polypeptide of the invention and a pharmaceutically acceptable carrier or excipient. Such carriers may include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol and combinations thereof. The formulation should suit the mode of administration.

- 10 The invention further relates to diagnostic and pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, reflecting  
15 approval by the agency of the manufacture, use or sale of the product for human administration.

Polypeptides, polynucleotides and other compounds of the present invention such as those identifiable by screening methods as described above may be employed  
20 alone or in conjunction with other compounds, such as therapeutic compounds.

The pharmaceutical compositions may be administered in any effective, convenient manner including, for instance, administration by topical, oral, anal, vaginal, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes among others.

- 25 The pharmaceutical compositions generally are administered in an amount effective for treatment or prophylaxis of a specific indication or indications. In general, the compositions are administered in an amount of at least about 10 µg/kg body weight. In most cases they will be administered in an amount not in excess of about 8 mg/kg body weight per day. Preferably, in most cases, dose is from about  
30 10 µg/kg to about 1 mg/kg body weight, daily. It will be appreciated that optimum

dosage will be determined by standard methods for each treatment modality and indication, taking into account the indication, its severity, route of administration, complicating conditions and the like.

- 5 In therapy or as a prophylactic, the active agent may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion, preferably isotonic.

10 Alternatively the composition may be formulated for topical application for example in the form of ointments, creams, lotions, eye ointments, eye drops, ear drops, mouthwash, impregnated dressings and sutures and aerosols, and may contain appropriate conventional additives, including, for example, preservatives, solvents to assist drug penetration, and emollients in ointments and creams. Such topical formulations may also contain compatible conventional carriers, for  
15 example cream or ointment bases, and ethanol or oleyl alcohol for lotions. Such carriers may constitute from about 1% to about 98% by weight of the formulation; more usually they will constitute up to about 80% by weight of the formulation.

20 For administration to mammals, and particularly humans, it is expected that the daily dosage level of the active agent will be from 0.01 mg/kg to 10 mg/kg, typically around 1 mg/kg. The physician in any event will determine the actual dosage which will be most suitable for an individual and will vary with the age, weight and response of the particular individual. The above dosages are  
25 exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

30 The present invention further provides a method of producing a protein of the invention, which method comprises introducing into an appropriate cell line a

vector comprising a polynucleotide as defined herein under conditions suitable for obtaining expression of the protein.

5 The present invention further provides a method of producing trimers comprising the protein of the invention, which method comprises introducing into an appropriate cell line a vector comprising a polynucleotide as defined herein under conditions suitable for obtaining expression of the protein, and allowing the protein produced to form into trimers.

10 As shown in Example 6, the protein of the invention binds to B cell lines such as the Burkitt Lymphoma cell line Raji, the B lymphoma cell line ROMI 8866 and PRMI8826. As shown in example 7, in preparations of whole blood, the protein of the invention binds only to B cells, not T cells. These examples confirm the presence of the receptor for the protein of the invention on B cells, and support  
15 the role of the protein of the invention in regulation of the immune system, and diseases as described above. This is also supported by the fact that the protein of the invention is strongly expressed in cells and tissues of the immune system as shown in example 5.

20 One of the first events induced by most members of the TNF ligand family is activation of NF- $\kappa$ B. Mukhopadhyay et al (Journal of Biological Chemistry Vol. 274 issue 23 June 4<sup>th</sup> 1999, pp 15978 – 15981) have demonstrated that the protein of the invention can activate NF- $\kappa$ B in a dose and time dependent manner. The range of doses used was 1pM to 1000pM. Treatment of cells with  
25 as little as 1pM of the protein of the invention produced an increase in NF- $\kappa$ B activation compared to untreated cells. The activation of this important transcription factor suggests that the protein of the invention may be involved in activation of inflammatory pathways. Molecules that modulate the interaction of the protein of the invention with its receptor will hence be able to modulate the  
30 activation of NF- $\kappa$ B and so will be useful in any diseases that are responsive to modulation of the level of activity of NF- $\kappa$ B, for example diseases of the immune system such as autoimmune disease, inflammatory diseases such as rheumatoid arthritis, neuronal inflammation and asthma and the proliferative diseases such as cancer. Mukhopadhyay et al further demonstrate that  
35 activation of NF- $\kappa$ B in the same system can be inhibited by antibodies specific to

the protein of the invention. According to Mukhopadhyay et al, the protein of the invention was incubated with the specific antibodies before being used to treat cells. In contrast to the previous experiment, reduced activation of NF- $\kappa$ B was observed.

5 It is postulated that the presence of the antibody affects the binding of the protein of the invention to its receptor, thus preventing generation of a signal and consequently reducing NF- $\kappa$ B activation. These findings indicate that other compounds, for example small molecules, which modulate the interaction between the protein of the invention and its receptor can be identified in a screen  
10 and can be used to modulate NF- $\kappa$ B activation and other downstream effects.

In Example 9, Chromosomal localisation experiments show that the gene encoding the protein of the invention maps to human chromosome 13, region q33. No other TNF ligand family members have been mapped to this region.  
15 Abnormalities in this locus have been characterised in Burkitt Lymphomas as the second most frequent defect (Berger et al Genes Chromosomes Cancer. 1:115-118). Also, as shown in Example 6, the protein of the invention binds strongly to the Burkitt lymphoma cell line Raji, and Schneider et al (as referenced above) have demonstrated that the soluble form of the protein of the invention binds  
20 strongly to other Burkitt Lymphoma cell lines such as BJAB, Namalawa, Ramos and JIYOYE.

Mukhopadhy et al (as referenced above) have demonstrated that the protein of the invention is able to inhibit the growth of human tumour cell lines. Activation of NF- $\kappa$ B is an early cellular response which is generally followed by cytotoxic  
25 effects to tumour cells. By treating various cell lines with the protein of the invention and examining them for viability, the authors were able to show that there is a dose dependent decrease in the viability of cells in the presence of the protein of the invention. This was demonstrated for a human histiocytic lymphoma cell line, a prostate cancer cell line, a colon cancer cell line, a cervical carcinoma cell line and a breast carcinoma cell line.  
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These facts suggest that the protein of the invention may play an important role in the regulation of tumour development, and that molecules that can modulate the interaction of the protein of the invention with its receptor may be useful in

the treatment of cancer. Also, the protein of the invention itself, in its membrane bound or its soluble form, may be useful in the treatment of cancer.

5 Schneider et al (Journal of Experimental Medicine volume 189 number 11 June 7<sup>th</sup> 1999 pp 1747-1756) have demonstrated that B cell growth can be costimulated by the full length protein of the invention (i.e. the membrane bound form) as well as by the soluble form of the protein of the invention. Hence either form can be utilised in therapy, or to form the basis of a screen for small molecules which can modulate the interaction between the protein of the  
10 invention and its receptor.

Brief Description of the Figures:

15 **Figure 1** shows sequence ID No. 1 - the amino acid sequence of the soluble human form of the protein of the invention. Receptor binding sites are shown in bold, and potential N-linked glycosylation sites are marked with a dot.

20 **Figure 2** shows sequence ID No. 2 - the amino acid sequence of the human membrane bound form of the protein of the invention, which comprises within it the soluble form of Seq ID No. 1. Annotations are as for figure 1. The transmembrane sequence is underlined.

25 **Figure 3** shows sequence ID No. 3 - the cDNA nucleotide sequence which encodes the amino acid sequence of Seq ID No. 1, aligned to the amino acid sequence of Seq ID No. 1. Receptor binding regions are boxed, and potential N-linked glycosylation sites are marked with a dot.

30 **Figure 4** shows sequence ID No. 4 - the cDNA nucleotide sequence which encodes the amino acid sequence of Seq ID No. 2, aligned with the amino acid sequence of Seq ID No. 2. Annotations are as for figure 3. The transmembrane sequence is underlined.

**Figure 5** shows sequence ID No. 5 - the amino acid sequence of the soluble mouse form of the protein of the invention. The N-linked glycosylation site is marked with a dot.

5     **Figure 6** shows sequence ID No. 6 - the amino acid sequence of the mouse membrane bound form of the protein of the invention. Annotations are as for figure 5. The transmembrane region is underlined.

10     **Figure 7** shows sequence ID No. 7 - the cDNA nucleotide sequence that encodes the amino acid sequence of Seq ID No. 5, aligned with the amino acid sequence of Seq ID No. 5. Annotations are as for figure 5.

15     **Figure 8** shows sequence ID No. 8 - the cDNA nucleotide sequence that encodes the amino acid sequence of Seq ID No.6, aligned with the amino acid sequence of Seq ID No. 6. Annotations are as for figure 6.

**Figure 9** shows an alignment between the mouse and human forms of the full length form of the protein of the invention. (Seq ID No. 2 and Seq ID No.6)

20     **Figure 10** Shows analysis of induction of CD40 in the presence (shaded) or absence (unshaded) of the recombinant soluble human form of the protein of the invention (A) or IL-4 (B).

25     **Figure 11** shows Northern Blot analysis of the tissue specific expression of the protein of the invention in normal mouse (A) and human tumour cell lines (B).(B) tissues.

30     **Figure 12** shows Northern Blot analysis of the tissue specific expression of the protein of the invention in immune related tissue (A) and human tumour cell lines (B).

35     **Figure 13** shows cell binding data of FLAG-sD7 (as defined in example 3 below). The shaded area indicates binding to the B-cell lymphoma cell line RPMI 8866. In the absence of FLAG-sD7 no binding is seen (dotted line)



**Figure 14** shows binding of the FLAG-sD7 to CD19+ Bcells (A) and CD3+ Tcells (B) in whole blood, demonstrating the specificity of binding, to B cells only.

5 **Figure 15** shows gel filtration of recombinant FLAG-sD7, and the subsequent SDS-PAGE analysis of the fractions shown to contain protein. These results indicate that the soluble form of the protein of the invention is able to trimerise.

Throughout the examples:

10 the protein having the amino acid sequence as shown in figure 1 will be termed soluble D7 ligand, and the protein having the amino acid sequence as shown in figure 2 will be termed D7 ligand.

Example 1: Use of soluble D7 ligand in a screen to identify compounds that modulate the interaction between the D7 ligand and its receptor.

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All incubations are done at room temperature

20 Costar RIA/EIA high binding plates are coated with goat anti-human IgG (Sigma I3382) at 2µg/ml in PBS overnight. The coating antibody is removed, and the plates are blocked for at least 2 hours in PBS/2% (w/v) BSA. Plates are then washed three times with PBS/0.1% (v/v) Tween20.

100µl receptor-Fc (1µg/ml) in PBS/1% (w/v) BSA/0.1% (v/v) Tween20 is added, and plates are incubated for 1 hour. Plates are washed five times with PBS/0.1% (v/v) Tween20.

25 Biotin-soluble D7 ligand dilutions in PBS/1% BSA/0.1% Tween20 are added, and plates are incubated for 1 hour. Plates are washed five times with PBS/0.1% (v/v) Tween20.

30 Streptavidin alkaline phosphatase (1:1000) (Amersham RPN1234) is added, and plates are incubated for 1 hour. Plates are washed five times with PBS/0.1% (v/v) Tween20.

Binding is detected using Life Technologies amplifier solutions (19589-019).

Example 2: A cell based screen to identify compounds that modulate the interaction between the soluble D7 ligand and its receptor

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A general protocol for using a cell based screen to identify compounds that modulate the interaction between the soluble D7 ligand and its receptor is as follows:

- 5 A B cell line known to bind and respond to the D7 ligand is treated with recombinant soluble human D7 ligand exemplified (e.g. FLAG-shD7 as exemplified below) for a defined time.

Cells are harvested, and the response assayed (The response may be possibly proliferation, apoptosis, NF- $\kappa$ B activation or cytokine production). The assay  
10 enables determination of whether the addition of compounds inhibits the induction of a response in target cells.

A specific example is as follows:

#### Soluble D7 ligand upregulates CD40

- 15 L3055 Burkitt's lymphoma cell line was grown on a feeder layer of human foetal fibroblast cells (HFF515) in L3 medium (RPMI 1640 + 10% Serum Supreme + antibiotics). HEK293 cells were grown in DMEM supplemented with 10% foetal calf serum and antibiotics. L3055 cells were treated either with control medium (four parts L3 medium plus one part HEK 293T cell supernatant) or with sD7  
20 medium (four parts L3 medium plus one part cell supernatant from HEK293T cells harvested 24 hours after transient transfection with sD7) or with IL-4 (control medium plus 200U/ml recombinant human IL-4 [Sigma]) and incubated at 37°C for 72 hours. Cells were harvested and washed once in binding buffer then stained with FITC-conjugated mouse anti-human CD40 (Transduction  
25 Laboratories) at room temperature. The cells were then washed twice in binding buffer before analysis by flow cytometry. Induction of CD40 was observed on treatment with sD7 for 72 hours compared to the control. This assay is repeated in the presence of a molecule which inhibits or increases the induction of the response in the target cells, and the results compared. Inhibition/increase of  
30 response can be clearly demonstrated. The end result of CD40 upregulation is that B cells are signalled for growth and differentiation. Thus, this experiment supports a role for the D7 ligand in the management of immune responses, and in diseases of the immune system such as inflammation.

Example 3: Synthesis and Purification of the soluble D7 ligand

Nucleic acid encoding the soluble human D7 ligand (amino acids 133 to 285) was generated by PCR using the cloned full-length open reading frame as a template.

Nucleic acid encoding the soluble human ligand D7 was cloned into vector pFLAG-CMV-1 (Kodak) (containing a CMV promoter, a preprotrypsin leader sequence, an amino-terminal FLAG epitope and a human growth hormone polyA addition sequence) to form construct pFLAG-CMV-1-hsD7.

$5 \times 10^6$  HEK 293T cells were resuspended in 250 $\mu$ l cytomix (120mM KCl; 0.15mM  $\text{CaCl}_2$ ; 10mM  $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ , pH 7.6; 25mM Hepes, pH 7.6; 2mM EGTA, pH7.6; 5mM  $\text{MgCl}_2$ ; 2mM ATP; 5mM glutathione; pH adjusted with KOH) containing 25 $\mu$ g pFLAG-CMV-1-hsD7. Transfection was carried out by using a BioRad gene pulser (960 $\mu$ F, 270V).

Following transfection, cells were left on ice for 10 min, then transferred to a 75cm<sup>2</sup> tissue culture flask containing 15ml medium (DMEM, 10% FCS, 2mM L-glutamine, penicillin (5 $\mu$ g/ml) and streptomycin (5 $\mu$ g/ml)). Medium containing secreted ligand was harvested after 48h and applied to an affinity chromatography column containing anti-FLAG M2 antibody coupled to agarose (Kodak). This was washed with Tris-buffered saline (pH 7.4) and fractions were eluted in 0.1M citrate buffer (pH 2.5). Fractions were immediately neutralised with 0.2 volumes 1M Tris.HCl (pH 7.6).

Fractions containing a human soluble D7 ligand linked to the FLAG epitope (FLAG-hsD7) were identified by Western blotting using M2 anti-FLAG antibody.

These fractions were pooled, and concentrated using a Centricon Plus-20 (NWML 5000) column (Millipore). FLAG-hsD7 ligand was stored at -70°

Example 4: Synthesis and Purification of the D7 ligand

The open reading frame of human D7 ligand is cloned into vector pcDNA3 (containing a CMV promoter and a bovine growth hormone polyA addition signal) to form construct pcDNA3-hD7.

Plasmid pcDNA3-hD7 is transiently transfected by electroporation into HEK 293T cells (protocol as in example 3).

Cells are harvested after 48h, and homogenised using a Dounce homogeniser in three volumes of protein extraction buffer (25mM Hepes pH 7.4, 0.5% Triton-X-100, 1 "complete" protease inhibitor cocktail tablet (Boehringer Mannheim) per 50ml buffer).

- 5 The D7 ligand is purified by affinity chromatography using anti-D7 antibody coupled to agarose.

Example 5: Northern Blot analysis of the tissue distribution of the D7 ligand.

- 10 cDNA coding for human D7 ligand was excised from pCDNA3-hD7 (see example 4) with the restriction enzymes BamH1 and Xba1. This cDNA fragment was labelled with <sup>32</sup>P dCTP using the Amersham ready-prime system according to the manufacturers protocol. A 5µl aliquot of this mixture was mixed with 10 ml Expresshyb solution (Clontech 8015-1) and the resulting mixture was incubated
- 15 with one of the following clontech blots: Mouse (7762-1), Human-1 (#7760), Human Cancer Cell line (#7757) or Human Immune System II (#7768-1); for 2 hours at 65°C with shaking. The probe solution was then removed and the blot was washed successively with 2X SSC (saline sodium citrate), 0.05% SDS at room temperature for three 20 minute periods. This was followed with one wash
- 20 with 0.1% SSC, 0.1% SDS at room temperature. The blot was then exposed to Kodak XAR-5 film at -70°C for 48 hours.

- The results of the Northern blot analysis show that D7 ligand RNA is expressed in heart, lung, spleen, kidney and skeletal muscle but not brain in both mice (figure 11A) and humans (figure 11B). A blot of immune-related tissues
- 25 demonstrates strong expression of D7 ligand RNA in spleen, lymph node, thymus, appendix, bone marrow and peripheral blood leukocytes (fig12A) supporting its potential role as a regulator of immune system functions. Analysis of RNA from a range of human tumour cell lines shows expression of D7 ligand RNA in HL-60 promyelocytic leukaemia cells but not in a range of other tumour
- 30 cell lines (Figure 12B). The presence of D7 ligand in a leukaemic cell line also supports the fact that the D7 ligand is involved in immune system regulation and disorders.

Example 6: Detection of cell surface binding of FLAG-sD7

10<sup>6</sup> cells were incubated with 50ng FLAG-hsD7 ligand (see example 3) in binding buffer (PBS/2.5% FCS/0.1% sodium azide) for 10 minutes at room temperature. After washing once in binding buffer, cells were incubated with 1µg anti-FLAG M2 antibody for 10 minutes at room temperature. Cells were washed once in binding buffer, then incubated with 150µg phycoerythrin-conjugated anti-mouse antibody for 10 minutes at room temperature. Following two further washes in binding buffer, flow cytometry was performed using a Coulter XL benchtop flow cytometer and data were collected on 10<sup>4</sup> viable cells.

Results of one such experiment are shown in figure 13. No significant signal was detectable when any of the lines tested were treated with anti-FLAG M2 antibody and R-phycoerythrin-conjugated second antibody only, but after prior treatment with FLAG-sD7, the signal clearly demonstrates that FLAG-hsD7 binds to the B lymphoma cell line RPMI 8866. Experiments with other cell lines have shown that FLAG-hsD7 binds two other B cell lines (RPMI 8226 and Raji) but does not bind to the T cell lines H9 and Jurkat, or the myelomonocytic lineage lines HL-60, U937 or THP-1. These results show that the extracellular domain of human D7 ligand binds to B cells, supporting its potential role in regulation of the immune system, and also suggesting that expression of the D7 receptor is restricted to B cells.

Example 7: Detection of cell surface binding of Flag-sD7 in whole blood.

Whole blood from healthy volunteers was diluted 1:10 with 3.8% (w/v) sodium citrate. 100 ul was used in each binding assay. Cell surface binding of FLAG-sD7 was detected as in example 6, except that the second antibody was Alexa 488-conjugated anti-mouse IgG (Molecular Probes) and, after one wash, cells were incubated with 1 µg PE-conjugated mouse anti-human CD3 (Becton Dickinson) or PE-conjugated mouse anti-human CD19 (Coulter-Immunotech). This experiment confirms the specificity of binding of FLAG-sD7 demonstrated in Example 6. From Figure 13 it can be seen that although both B cells and T cells were present, FLAG-sD7 bound only the CD19<sup>+</sup> Bcells (Figure 14 A), and not the CD3<sup>+</sup>Tcells (Figure 14 B). This confirms the specificity of binding to B cells

seen with cell lines, and again suggests that expression of the D7 receptor is restricted to B cells.

Example 8: FLAG-sD7 is able to trimerise.

5 Purified recombinant FLAG-sD7 was fractionated on a Superose 12 column (Pharmacia). Proteins were eluted in PBS and fractions (1ml) were analysed by Western blotting using anti-FLAG M2 antibody (Sigma). The column was calibrated with standard proteins: apoferritin (443 kDa), b-amylase (200kDa), ADH (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C  
10 (12.5 kDa). Figure 15 A shows the eluate from the column. Figure 15 B shows the fractions containing the peaks seen in Figure 15A run on an SDS-PAGE. The SDS-PAGE markers are on the left hand side, indicating that the denatured protein runs at approximately 22kDa. The markers on the top of the gel are the standard proteins used to calibrate the column, and they show that FLAG-sD7  
15 elutes in the gel filtration fractions corresponding to a molecular weight of approximately 70 to 25 kDa. These experiments demonstrate that FLAG-sD7 is able to assemble correctly into a homotrimer, with molecular weight approximately 3 x 22 kDa.

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Example 9 FISH mapping of the D7 ligand.

Lymphocytes isolated from human blood were cultured in a-minimal essential medium (a-MEM) supplemented with 10% foetal calf serum and phytohemagglutinin at 37°C for 68 – 72 hours. The lymphocyte cultures were  
25 treated with BrdU (0.18mg/ml Sigma) to synchronise the cell population. The synchronised cells were washed three times with serum free medium to release the block and recultured at 37°C for 6 hours in a-MEM with thymidine (2.5µg/ml Sigma). Cells were harvested and slides were made by using standard procedures including hypotonic treatment, fixation and air-dry.  
30 Slides were baked at 55°C for 1 hour. After RNase treatment, the slides were denatured in 70% formamide in 2xSSC for 2 min at 70°C followed by dehydration with ethanol. D7-27 DNA probe which is the full length D7 cDNA as shown in figure 4, plus 150 nucleotides of 5' untranslated region and 50 nt of 3' untranslated region) was biotinylated with dATP, and probes were denatured at  
35 75°C for 5 min. in a hybridisation mix consisting of 50% formamide and 10%

5 dextran sulphate. Probes were loaded on the denatured chromosomal slides. After over night hybridisation, slides were washed and detected as well as amplified. FISH signals and the DAPI banding was recorded separately by taking photographs, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposing FISH signals with DAPI banded chromosomes. The DAPI banding showed that the signal mapped to human chromosome 13, and the FISH results further mapped it to region q33.